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Studies on the Excystation of Different Species of *Eimeria* in vitro

H.D. Chapman

Houghton Poultry Research Station, Houghton, Huntingdon, Cambs. England

Summary. The role of enzymes in the excystation of an embryo-adapted strain of *E. tenella* (TA) was investigated; 0.1% crude trypsin or pure trypsin in combination with 0.5% bile salt resulted in 75% and 58% excystation respectively. Both enzymes were more effective than chymotrypsin. Addition of 0.01% chymotrypsin to 0.1% purified trypsin resulted in 76% excystation suggesting that both trypsin and chymotrypsin may be important for excystation in vitro. Pre-incubation of sporocysts in 2% bile salt prior to the addition of enzyme resulted in lower excystation. Sporozoites released at various enzyme concentrations did not differ in infectivity when inoculated into chicken embryos.

Excystation of the Houghton strains of *E. acervulina, E. praecox* and *E. maxima* occurred more rapidly at lower concentrations of crude trypsin plus 0.5% bile salt than the Houghton strains of *E. brunetti* or *E. tenella.* Sixty per cent of sporozoites of *E. acervulina* had excysted after 25 min incubation with 0.001% crude trypsin plus 0.5% bile salt. At the same concentrations 72% *E. praecox* had excysted after 25 min, 43% *E. maxima* after 60 min, 20% *E. brunetti* after 105 min and 1% *E. tenella* after 105 min. Maximum numbers of sporozoites of *E. brunetti* and *E. tenella* were found after 105 min incubation with 0.1% crude trypsin.

Whereas incubation of *E. acervulina, E. praecox* and *E. maxima* for 105 min in 0.01% crude trypsin resulted in sporozoite lysis, no lysis was found with *E. brunetti* or *E. tenella* after incubation in crude trypsin.

Introduction

Preparation of large numbers of infective sporozoites is a prerequisite for in vitro studies with *Eimeria.* The excystation of sporozoites has been studied by several investigators. Doran and Farr (1962) for example, used crude trypsin combined with sodium taurocholate to excyst sporozoites of a variety of species

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of *Eimeria*. In this laboratory a similar procedure has been used to obtain sporozoites of *E. tenella* (Long, 1970). Sporocyst suspensions are incubated at 41° C in 0.25% crude trypsin (1:250 powder, Difco) and 0.5% bile salt (Difco). After 90 min 60–80% of the sporozoites are released from the sporocysts.

Recently Wang and Stotish (1975) obtained varying yields of sporozoites of *E. tenella* when sporocysts were pre-incubated in 2% sodium taurocholate followed by treatment with crude trypsin. Furthermore, they found that some batches of crude trypsin, when used at 0.5%, were capable of lysing sporozoites. They concluded that chymotrypsin may be the only essential enzyme for in vitro excystation of *E. tenella* and suggested that the action of crude trypsin may be explained by the fact that chymotrypsin is a common contaminant in crude pancreatic enzyme samples.

The purpose of this study was to investigate the role of enzymes in the excystation of different species of *Eimeria*. For studies on different enzymes an adapted strain of *E. tenella* (TA) which had been passaged 70 times in chicken embryos was used. This strain shows excellent reproduction in the embryo (Long, 1972); infectivity of sporozoites could therefore be quantified using oocyst production in embryos as an index of infection. Comparable infections of chickens involve either transfer of sporozoites via gelatin capsules, or surgery to expose the intestine (Shirley and Millard, 1976). Studies were also carried out to determine the optimum conditions for excystation of the Houghton strains of *E. acervulina, E. praecox, E. maxima, E. brunetti* and *E. tenella* obtained from chickens.

Materials and Methods

Infective Material

Oocysts of the adapted strain of *E. tenella* (TA) were obtained from chicken embryos. Full details of the experimental methods for the maintenance of embryos, collection of oocysts etc., have been given by Long (1972). Oocysts of the Houghton strains of *E. acervulina, E. praecox, E. maxima, E. brunetti*, and *E. tenella* were harvested from the faeces of birds by the standard methods used in this laboratory and sporulated in a 5% solution of potassium dichromate. After removal of potassium dichromate, oocysts were treated with a commercial solution of sodium hypochlorite giving a final concentration of 1–1.4% to produce oocyst suspensions free from bacteria. They were then washed by repeated centrifugation and suspended in Hanks balanced salt solution (HBSS). Sporocysts were released from the oocysts by shaking (Whirlimix, Fisons Ltd.) in a bottle containing 0.5 mm diam. glass beads (Jencons Ltd.) for approximately 20 s.

Excystation of the Adapted Strain of E. tenella. In the first experiment sporocysts were incubated at 41° C for 90 min with a mixture of different enzymes plus 0.5% bile salt. In the second experiment the procedure described by Wang and Stotish (1975) was followed. Sporocysts were pre-incubated for 20 min with 2% bile salt, the salt was removed by sedimentation and excystation completed by incubating with enzymes alone. Each experiment was repeated three times and the results combined. After 90 min incubation using different concentrations of enzymes plus bile salt, sporozoites excysted (Experiment 1) were inoculated into chicken embryos. The incubation medium was centrifuged at 1000 *g* for 5 min, the supernatant was discarded and the sporozoites suspended in fresh HBSS and counted. Doses of 5000 sporozoites obtained from each treatment were inoculated into six groups of five 11-day old embryos. Six days after inoculation oocysts were recovered from each group of embryos and counted.

Excystation of E. acervulina, E. praecox, E. maxima, E. brunetti, and E. tenella. Sporocysts were incubated with different concentrations of crude trypsin and bile salt. Percentage excystation was recorded at different times during incubation. Treatments were duplicated in each experiment and the results combined. In all experiments percentage excystation was based upon the initial number of sporocysts and the number of sporozoites released. Since each sporocyst contains two sporozoites then twice that number of sporozoites was equivalent to 100% excystation (Wang and Stotish, 1975).

Materials. Crude trypsin (1:250) and bile salt were obtained from Difco Ltd., α chymotrypsin (C4129), purified from bovine pancreas, and purified trypsin (T1005, treated with diphenyl carbamyl chloride to inactivate any chymotrypsin usually present in trypsin preparations), were obtained from Sigma Chem. Co. Ltd. After addition of enzymes to HBSS the pH was adjusted to 7.4–7.6 by addition of 1% $NaHCO_3$.

Results

The Effect of Different Enzymes on Excystation of the Adapted Strain of E. tenella

Experiment 1. The results obtained when sporocysts were incubated with a mixture of enzymes plus bile salt are shown in Table 1. Maximum numbers of sporozoites were found after 90 min incubation. Crude trypsin and pure trypsin at 0.1% resulted in 75 and 58% excystation respectively. When lower concentra-

Table 1. The effect of different enzymes on the excystation of sporozoites of the adapted strain of *E. tenella* (TA)

Enzyme concentration (%)		Expt. 1 – Enzyme + 0.5% bile salt		Expt. 2 – Enzyme alone[a]	
		45 min	90 min	45 min	90 min
		% Excystation			
Crude trypsin	1[b]	53 ± 2.9	84 ± 5.7	55 ± 14.1	84 ± 12.7
	0.1	31 ± 1.1	75 ± 2.6	34 ± 13.9	50 ± 10.9
	0.01	3 ± 0.4	44 ± 1.2	10 ± 4.3	19 ± 4.8
	0.001	1 ± 0.1	1 ± 0.3	0	1 ± 0.2
Pure trypsin	1[b]	32 ± 4.4	52 ± 2.7	21 ± 7.9	37 ± 6.7
	0.1	17 ± 2.9	58 ± 4.1	15 ± 7.0	21 ± 4.3
	0.01	2 ± 0.6	12 ± 1.8	2 ± 0.8	4 ± 1.1
	0.001	1 ± 0.4	1 ± 0.2	0	1 ± 0.3
Chymotrypsin	1[b]	2 ± 0.9	31 ± 3.3	6 ± 2.8	8 ± 2.1
	0.1	0	1 ± 0.1	4 ± 1.7	6 ± 1.5
	0.01	0	0	0	2 ± 0.5
Pure trypsin + chymotrypsin	0.1 + 0.01	14 ± 0.9	76 ± 6.6	–	–

[a] Pre-incubated for 20 min in 2% bile salt

[b] No sporozoite lysis after 180 min incubation

± Standard error of the mean

No excystation occurred when sporocysts were incubated in medium or bile salt alone

tions were used excystation was reduced. Excystation was not so great when chymotrypsin was used, only 31% of sporozoites were released at a concentration of 1%. When pure trypsin and chymotrypsin were combined at concentrations of 0.1 and 0.01% respectively 76% excysted. No excystation occurred when crude trypsin (0.1%), pure trypsin (0.1%) or chymotrypsin (1%) were used in the absence of bile salt. There was no evidence of lysis of sporozoites after 180 min incubation at the concentrations tested.

Experiment 2. The results obtained when sporocysts were pre-incubated with 2% bile salt are shown in Table 1. Excystation did not occur more rapidly as a result of pre-incubation with bile salt, excystation had commenced by 45 min but maximum numbers of sporozoites were not found until 90 min incubation. Crude trypsin and pure trypsin at 0.1% resulted in 50 and 21% excystation respectively. Chymotrypsin was not so effective, only 8% of sporozoites were relased at a concentration of 1%.

Oocyst Production of E. tenella (TA) in Embryos Given 5000 Sporozoites Excysted at Different Concentrations of Enzyme Plus 0.5% Bile Salt

Oocyst production in embryos given sporozoites excysted with 1% crude trypsin, pure trypsin or chymotrypsin was 4.6 ± 0.6, 4.2 ± 0.7, 4.7 ± 0.8/ embryo respectively. Oocyst production in embryos given sporozoites excysted with 0.01% crude trypsin or pure trypsin was 4.8 ± 0.4 and 5.5 ± 0.4 respectively. No significant difference in oocyst production was found between any of the sporozoite inocula.

The Effect of Crude Trypsin and Bile Salt on the Excystation of Different Species of Eimeria

Results with *E. acervulina, E. praecox* and *E. maxima* are shown in Table 2. Large numbers of sporozoites of *E. acervulina* and *E. praecox* excysted after 10 min incubation with 0.01% crude trypsin plus 0.5% bile salt and by 25 min excystation was complete. Excystation of *E. maxima* at these concentrations was not so rapid and reached a peak of 51% after 60 min. All three species showed reduced excystation with lower concentrations of enzyme or bile salt. Prolonged incubation (105 min) at higher enzyme concentrations resulted in lysis of sporozoites. Small numbers of *E. acervulina* and *E. praecox* excysted without bile salt.

Results with *E. brunetti* and *E. tenella* are shown in Table 3. Excystation of *E. brunetti* and *E. tenella* had commenced after 25 min with 0.1% crude trypsin plus 0.5% bile salt but maximum numbers of sporozoites were not found until 105 min incubation. At lower concentrations of enzyme or bile salt excystation was reduced. Lysis of sporozoites was not observed after 105 min incubation.

Table 2. The effect of crude trypsin and bile salt on the excystation of sporozoites of the Houghton strains of *E. acervulina*, *E. praecox* and *E. maxima*

Concentration %		*E. acervulina*			*E. praecox*			*E. maxima*		
Crude trypsin	Bile salt	10 min	25 min	105 min	10 min	25 min	105 min	25 min	60 min	105 min
		% Excystation								
0.01	0.5	60	100	77[a]	80	96	28[a]	46	51	33[a]
0.005	0.5	74	100	69[a]	76	79	39[a]	30	50	35[a]
0.001	0.5	26	60	68	33	72	64	14	43	41
0.0001	0.5	0	0	11	0	4	0	0	0	0
0.005	0.1	51	63	46	25	40	0	3	8	17
0.005	0.01	5	9	18	1	7	3	1	3	3
0.005	–	3	4	12	0	3	1	0	1	0

[a] Sporozoite lysis

Table 3. The effect of crude trypsin and bile salt on the excystation of sporozoites of the Houghton strains of *E. brunetti* and *E. tenella*

Concentration %		*E. brunetti*			*E. tenella*		
Crude trypsin	Bile salt	25 min	60 min	105 min	25 min	60 min	105 min
		% Excystation					
0.1	0.5	39	54	73	6	90	89
0.01	0.5	21	55	72	0	21	69
0.005	0.5	6	15	47	0	6	19
0.001	0.5	4	7	20	0	0	1
0.1	0.1	16	32	37	3	29	65
0.1	0.01	0	1	2	0	5	21
0.1	–	0	0	0	0	0	0

Discussion

According to Wang and Stotish (1975) chymotrypsin is the essential enzyme responsible for excystation of *E. tenella*. They considered that the successful results achieved in experiments using crude pancreatic trypsin may be due to contamination of such preparations with chymotrypsin. It is unlikely however, that chymotrypsin alone could explain the action of crude trypsin since a high level of chymotrypsin (1%) was required to achieve >60% excystation in their experiments. In this study, excystation of the adapted strain of *E. tenella* with 0.1% crude or purified trypsin, plus 0.5% bile salt was 75% and 58% excystation respectively. Both enzymes were more effective than chymotrypsin. Addition of 0.01% chymotrypsin to 0.1% purified trypsin resulted in 76% excystation. This suggests that both trypsin and chymotrypsin may be important for the

excystation process in vitro. A similar conclusion was reached by Doran (1966) who reported that the combined effect of purified trypsin and α chymotrypsin might be responsible for the high excystation of *E. acervulina*.

Wang and Stotish (1975) incubated sporocysts in 2% sodium taurocholate for 20 min prior to addition of enzymes. In this study the number of sporozoites released as a result of pre-incubation with 2% bile salt was not as great as when the enzymes were combined directly with 0.5% bile salt. In the chicken both pancreatic secretion and bile are emptied via the ducts which open onto a common papilla on the wall of the duodenum. Sporocysts may therefore be exposed to both secretions simultaneously in vivo.

Excystation takes place following breakdown of proteins in the stieda body of the sporocyst. Some sporozoites may be released when enzymes are used alone but the process is hastened by the presence of bile salts. Wang and Stotish (1975) used 2% sodium taurocholate to achieve this, but results in these experiments suggest that such a high concentration may not be necessary. One advantage of crude trypsin in laboratories where these chemicals are used routinely, is its relative cost compared to pure chymotrypsin. Lysis of sporozoites was not seen when the adapted strain of *E. tenella* was treated with crude trypsin. Furthermore, there was no difference in infectivity of sporozoites excysted with various enzyme and bile salt concentrations. Crude trypsin was therefore used in subsequent experiments with other species of *Eimeria*.

Excystation of the Houghton strains of *E. acervulina, E. praecox* and *E. maxima* occurred more rapidly at lower concentrations of crude trypsin plus 0.5% bile salt than the Houghton strains of *E. brunetti* or *E. tenella*. According to Crompton and Nesheim (1976) and Long and Speer (1976), the time of excystation probably plays an important role in determining the site of sporozoite penetration of cells and their subsequent development in a particular region of the intestinal tract. Farr and Doran (1962) found that two duodenal species of *Eimeria* (*E. acervulina* and *E. meleagrimitis*) excysted in less time and farther anteriorly in the small intestine than two caecal species (*E. tenella* and *E. gallopavonis*). They also found that sporozoites of the caecal species survived longer in a medium containing 0.25% trypsin plus 1% sodium taurocholate or 5% bile. In this study 60% of sporozoites of *E. acervulina* had excysted after 25 min incubation with 0.001% crude trypsin plus 0.5% bile salt. At the same enzyme and bile salt concentrations 72% *E. praecox* excysted after 25 min, 43% *E. maxima* after 60 min, 20% *E. brunetti* after 105 min and 1% *E. tenella* after 105 min. Maximum numbers of sporozoites of *E. brunetti* and *E. tenella* were found after 105 min incubation with 0.1% crude trypsin. *Eimeria praecox* and *E. acervulina* excysted more rapidly than *E. maxima*; whereas the former species develop in the anterior of the small intestine, *E. maxima* develops in the mid-region of the gut (Crompton, 1976). These three species excysted more rapidly than either *E. brunetti* or *E. tenella* which live in the lower intestine and caecum respectively.

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